eISSN: 1984-3909
Coffee Science, e161863, 2021

Doi: https://doi.org/10.25186/cs.v16i.1863

Young coffee leaves biotransformed by aspergillus oryzae in enriched source of caffeic acid

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Received in December 21, 2020 and approved in September 20, 2021

ABSTRACT

This research focused on the study of young coffee leaves as a potential source of caffeic acid from the biotransformation of abundant chlorogenic acid. *Aspergillus oryzae* was isolated from koji rice and used for the solid-state fermentation of coffee leaves. The bioconversion of chlorogenic acid into caffeic acid was measured by HPLC-DAD. The water infusions of fermented coffee leaves were analyzed by sensory evaluation in comparison with unfermented coffee leaves as well as roasted coffee beans. The biotransformation of young coffee leaves yielded over 3.5-fold change increase of caffeic acid natural content in 87 hours, and equivalently, decreasing concentrations were observed for chlorogenic acids. Other bioactive compounds, such as mangiferin, rutin and caffeine, showed relatively minor changes. Sensory evaluation pointed out the effect of increasing caffeic acid-concentration on bitterness and astringency, which would have to be considered in the development and optimization of functional foods. As bitterness was increased by the enhanced caffeic acid concentrations, desirable flavor descriptors were also associated with fermented leaves, with the inclusion of notes traditionally associated with koji. This bioprocess proved to be highly efficient and could be implemented with relatively simple technology *in-farm* production systems.

Key words: Coffee leaves; Coffee biomass; Caffeic acid; *Aspergillus oryzae*.

1 INTRODUCTION

Caffeic acid has been extensively studied and associated with well recognized functionalities such as: cellular antioxidant (Colina et al., 2019; Spagnol et al., 2019), cytotoxicity (Matejczyk et al., 2018), anti-cancer (Brautigan et al., 2018; Khan; Maalik; Murtaza, 2016; Parzonko; Kiss, 2019; Singh et al., 2018), apoptosis (Chang et al., 2010), anti-microbial (Lima et al., 2016; Ogawa et al., 2018; Shen; Wang; Zuo, 2018), anti-viral (Langland et al., 2018), reduction of hepatic steatosis (Kim et al., 2018), and neuroprotective effect (Alzheimer's disease), (Chang et al., 2019).

However, caffeic acid is mostly found as a degradation product of chlorogenic acid, which is one of the most common hydroxycinnamic phenolic in dietary sources, particularly in coffee. It has been estimated that in average a 200 ml cup of arabica contains $7e^{-5}$ – 20 e^{-5} kg of chlorogenic acid, which for years has driven special research efforts in coffee's biological effects (Clifford, 1999). It has been also found that 33 % of chlorogenic acid is absorbed in the intestines, and the remaining 67 % is metabolized to caffeic acid in the colon, whereas 95 % of caffeic acid can be absorbed intact in the intestines (Olthof et al., 2003). Thus, detachment of the caffeic moiety from chlorogenic acid could enhance its absorption while preserving essential functionalities (Cao et al., 2019; Jackson et al., 2017). Moreover, considering other potential applications of caffeic acid, its skin absorption has been demonstrated (Santos et al., 2019).

With the prospective benefits on human health in mind, several authors analyzed a diversity of sources of caffeic acid other than coffee, with reports of $1e^{-9}$-$5.7e^{-7}$ kg.100 mL^{-1} in herbal infusions (Meinhart et al., 2019a), and up to 3.5 e^{-4} $kg.kg^{-1}$ (dry basis) in bilberry (Meinhart et al., 2019b). Alternatively, engineered microorganisms have been used for the biosynthesis of caffeic acids (Hernández-Chávez et al., 2019; Liu et al., 2019). Nevertheless, studies on the hydrolysis of chlorogenic acids for the production of caffeic acid from biomass are limited with few recent studies on green coffee beans (Siqueira Palmieri et al., 2018; Soares Bertges et al., 2020).

Coffee byproducts, naturally enriched with a diversity of phytochemicals with multiple functionalities, have been studied as promising resources with potential applications in the food and pharmaceutical industries (Chen, 2019; Gemechu, 2020; Klingel et al., 2020). Particularly, coffee leaves have been well recognized by both traditional culture and modern research for the potential benefit on human health (Monteiro et al., 2020; Novita et al., 2018; Segheto et al., 2018). More recently, coffee leaves were found to have higher values of total phenolic content as well as greater antioxidant capacity than roasted beans, and in general young leaves of coffee species were found to have similar quantities of chlorogenic acid than those in the green coffee beans (Acidri et al., 2020; Monteiro et al., 2020). In consideration of novel agricultural practices, coffee leaves are faster to grow than coffee beans, with several cycles in one year. The maturity of leaves and agroecological conditions could be managed towards the production of optimal concentrations of chlorogenic acid (Monteiro et al., 2020).

As pointed out, chlorogenic acid is extensively metabolized into compounds with lower antioxidant activity before it enter the circulation (Olthof et al., 2003), whereas caffeic acid is widely recognized as a bioactive compound efficiently absorbed in the intestines. Hence, cleavage of chlorogenic acid into caffeic acid represents a strategy for an enhanced absorption or applications of a well-recognized bioactive moiety. *Aspergillus* sp. has been used for the enhancement of phenolic contents by the hydrolysis of bound compounds, with a resulting increment of antioxidant properties in a diversity of biomasses (Gulsunoglu et al., 2020; Siqueira Palmieri et al., 2018; Torres-León et al., 2019). However, the effects of incrementing the concentration of free phenolic acids on sensory profiles, such as caffeic acid, has not been published. Thus, we aimed to evaluate the use of *Aspergillus oryzae* for the efficient biotransformation of young coffee leaves towards the production of a caffeic acid enriched tea with attractive sensorial properties.

2 MATERIAL AND METHODS

2.1 Young coffee leaves

Coffee leaves from *Coffea arabica* were obtained from a plantation at Santa Lucia experimental farm at National University (UNA, Costa Rica) located at 1250 MASL with an annual average temperature of 19 ˚C, andisol soils, and grown under partial shade. Only the youngest four leaves were collected, freeze dried at -80 ˚C for 24 hours, and ground into a fine powder able to pass through a #20 mesh.

2.2 Aspergillus oryzae fermentation

Aspergillus oryzae was isolated from koji rice (Marusan, Japan) by placing 6 grains per petri dish with PDA agar (Oxoid, England) and 2 drops of lactic solution (25 % in water v/v) (Honeywell Fluka, Germany). The isolates were grown at 30 °C for 7 days and the culture was confirmed by macromorphological observations. 10 mL of freshly prepared saline solution (0.8% NaCl) was used to suspend the spores, the concentration was adjusted by Neubauer chamber to 10^7 spores.mL^{-1}.

0.001 L of water was added to 0.001 kg of lyophilized ground coffee leaves in glass bottles, sterilized in autoclave (110 °C for 3 minutes), and inoculated with the 1 mL of the *Aspergillus oryzae* solution. For the control group 0.001 L of sterilized water was used. Fermentation was done in triplicates at 30 °C for 15, 25, 40, 50, 87, 120, and 160 hours.

2.3 Extraction and HPLC analysis

Phenolic compounds from each glass bottle containing 0.001 L of water + 0.001 kg of lyophilized ground coffee leaves + *A. oryzae* were extracted with a solution of water-methanol (HPLC grade ≥99.9%, Sigma Aldrich, Germany) (1:1)) acidified with 1% acetic acid (J.T. Baker, USA). Extractions were performed on the whole fermented samples repeating 3 times with 0.01 L in ultrasonic bath for 20 minutes and centrifuged at 7000 rpm for 10 minutes. All three supernatants were collected in one single conical tube which was adjusted to a final volume of 0.03 L with the extraction solution. Samples of 2 mL from the collected supernatant were taken, filtered (0.45 µm, Sartorius Stedim Biotec, Germany), kept in HPLC vials, and stored at 2-5 °C before analysis.

For HPLC analysis an Agilent series 1200 was used, connected to autosampler, quaternary pump, and Diode Array Detector (DAD). The separation took place using a Zorbax Eclipse Plus C18 (4,6 x 100 mm, 3,5 µm, Agilent). The mobile phase was A) Millipore Milliq water (1% acetic acid), and B) acetonitrile (suitable for HPLC ≥99.9%, Fluka, Germany).

Samples were injected (20 µL) and eluted with a flow rate of 1 ml/min using the following conditions: 100% A for 10 min, 75 % A for 5 min, 50% A for 2 min, and 100% B for 1 min, with 1 min postconditioning. Detection was done at λ 275.4 nm and λ 325.5 nm.

A calibration curve was created using the following Sigma-Aldrich standards: caffeic acid (purity ≥98%), chlorogenic acid 5-CQA (purity ≥95%), caffeine (purity ≥99%), at concentrations of 25, 50, 100, 150, and 200 $\mu g.mL^{-1}$ (in water-methanol, 1:1, HPLC ≥99.9%, Sigma Aldrich, Germany).

2.4 Sensory analysis

Three water infusions were prepared to compare the taste perception between commercially available ground coffee, freeze dried coffee leaves and freeze-dried fermented coffee leaves.

The hot water infusions of coffee leaves and ground roasted coffee were prepared as follows: freeze dried coffee leaves or coffee were subjected to extraction with hot water (90 ˚C) for 20 minutes, at a ratio of 0.006 kg of powder to 0.1 L of water. The extract was filtered with Whatman® No. 4 filter paper.

The sensory panel was composed of 12 men and 8 women, for a total of 20 tasters, with ages between 20 and 52 years old. The panel members had experience in describing organoleptic profiles of coffee and attended training sessions for the standardization of taste descriptors. The panelists rated the intensity of different descriptors such as sweet, bitter, fermented (umami), astringent, and earthy on an evaluation sheet showing a 6-point numerical scale (0= absent, 5=very intense). They also described other perceived flavors. 5 ml of sample were used for every individual taste, being hold for 15 seconds and spitted afterwards. Water was used to rinse palate in between samples.

2.5 Statistical analysis

Results were expressed as means ± standard deviation. Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The differences were considered statistically significant at $P < 0.05$.

For sensory evaluation, a 6-point numerical scale (0 to 5) was used, with 0 indicating absent, and 5 very intense. Values were further normalized in a scale 0 to 1.

3 RESULTS

3.1 Effect of *Aspergillus oryzae* growth on caffeic acid concentration

The growth of *Aspergillus oryzae* (isolated form koji rice) on the coffee leaf powder was visually observed from 24 hours and noticed with abundant mycelium at 160 hours (Figure 1). For the purposes of this work, a purified culture and the elimination of interactions from unknown organisms were required, thus the isolation process was accompanied by macro morphological analysis. The design of these experiments was based on a solid-state fermentation for evaluating conditions inspired on the traditional techniques of koji fermentation, aiming at developing simplified agro-industrial techniques. Consequently, no mineral supplementation was used for the fermentation of coffee leaves.

Additionally, special attention was given to the specific release of caffeic acid from chlorogenic acid. It was observed by HPLC that the growth of *Aspergillus oryzae* caused a degradative hydrolysis of chlorogenic acid with the consequent increase of caffeic acid concentration. No changes in concentrations were observed for the control group (Figure 2).

As seen in Figure 2, very selective biotransformations took place caused by the growth of the isolated *Aspergillus oryzae*, with relatively minor changes, although significant losses, for other compounds with UV absorbance at 275 and 325 nm. From HPLC chromatograms chlorogenic acid was detected at 7.10 min, caffeine at 7.40 min, caffeic acid at 7.90 min, mangiferin at 8.10 min, and rutin at 11.40 min. Only the inverse relationship between chlorogenic acid by caffeic acid was significant.

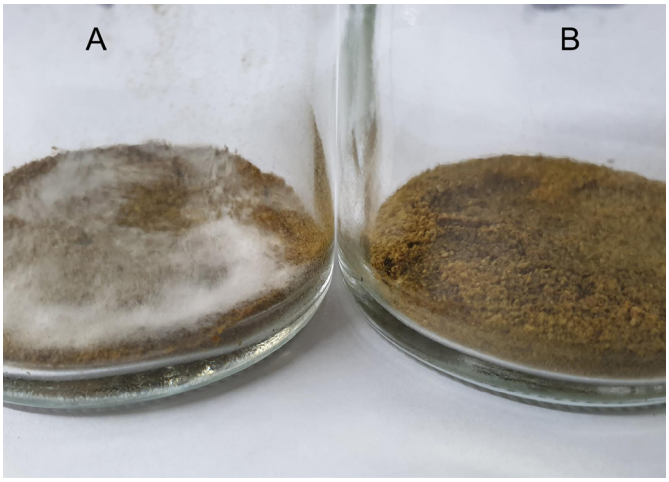

Figure 1: (A) young coffee leaves after 160 hours of solid-state fermentation with *Aspergillus oryzae* at 30 ºC, and (B) unfermented young coffee leaves (control).

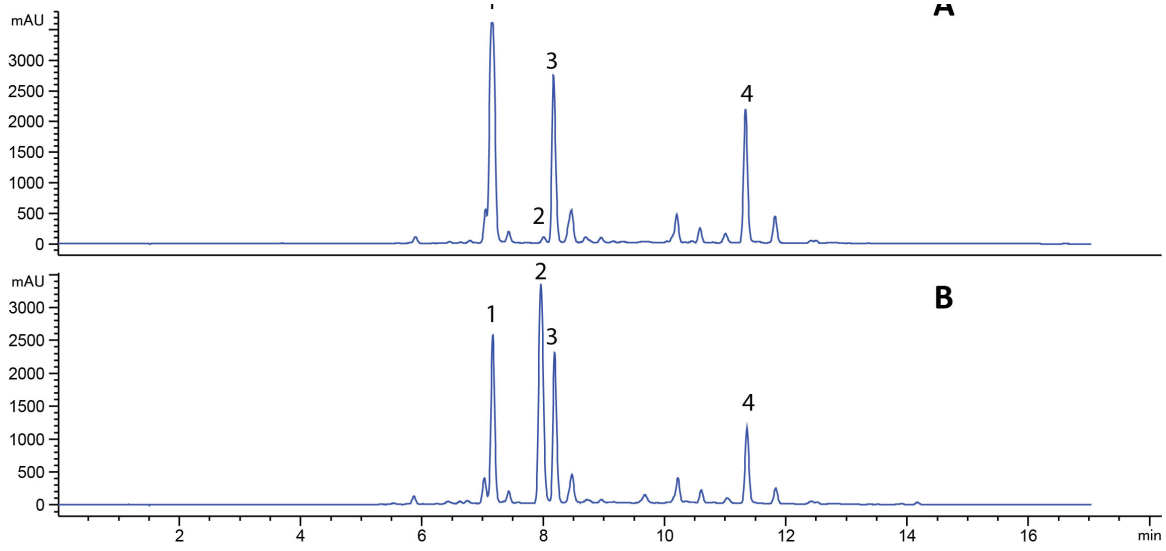

Figure 2: HPLC Chromatogram at 325 nm for (A) young lyophilized, unfermented coffee leaves and (B) young coffee leaves after 160 hours of solid-state fermentation with *Aspergillus oryzae* at 30 ˚C. (1) Chlorogenic acid, (2) caffeic acid, (3) mangiferin, (4) rutin.

It is important to highlight that small increases of caffeic acid with losses of chlorogenic acid were attributed to sterilization with autoclave under the conditions of the experiment (hydrated samples at 110 ˚C for 3 min). This condition caused increases of caffeic acid up to 5 %; yet, this transformation did not continue through the course of the experiments, as observed in the control group.

The growth of *Aspergillus oryzae* effectively induced the hydrolysis of chlorogenic acid to caffeic acid, before 15 hours, the values for caffeic acid and chlorogenic acid were 0.00379 ±6e^{-5} kg.kg-1 and 0.002076±0.00033 kg.kg^{-1} respectively; after 160 hours caffeic acid concentration increased to 0.01402±0.00106 kg.kg^{-1}, while the amount of chlorogenic acid dropped to 0.01229±0.00079 kg.kg^{-1} (Figure 3). Noteworthy, significant high conversions were achieved at 87 hours with concentrations of caffeic acid and chlorogenic acid 0.01292± 0.0007 kg.kg^{-1} and 0.01665± 0.00084 kg.kg^{-1} respectively.

3.2 Sensory profile

The effect of fermentation on sensory profiles were also evaluated, results are shown in Figure 4. The analysis compared tea of fermented and unfermented young coffee leaves and included black coffee for reference. In all cases the same solid (weight) to water (volume) ratio, and brewing time, was used. The predominant perceived effect was a significant increased bitterness on fermented leaves and equivalently an increased perceived astringency. This increased bitterness and astringency corresponded with the increasing concentration of caffeic acid, as follow: fermented leaves > unfermented > black coffee. This work also collected preliminary information regarding other perceived descriptors. Tea of unfermented leaves were naturally associated with a predominant grass/herb flavor and aroma. Fermented leaves were consistently associated with honey and fermented intense aroma.

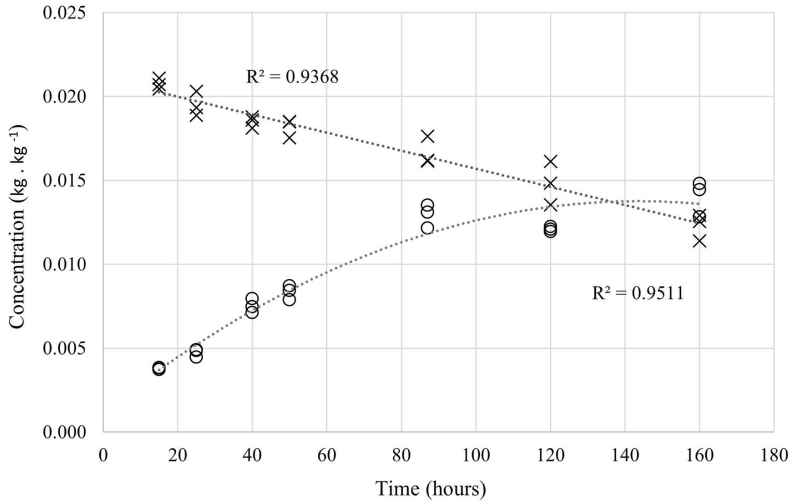

Figure 3: Concentration changes of chlorogenic acid (X) and caffeic acid (O) in young coffee leaves subjected to *Aspergillus oryzae* solid state fermentation. Values were calculated based on concentration changes in kg.kg^{-1} measured by RP-HPLC-UV.

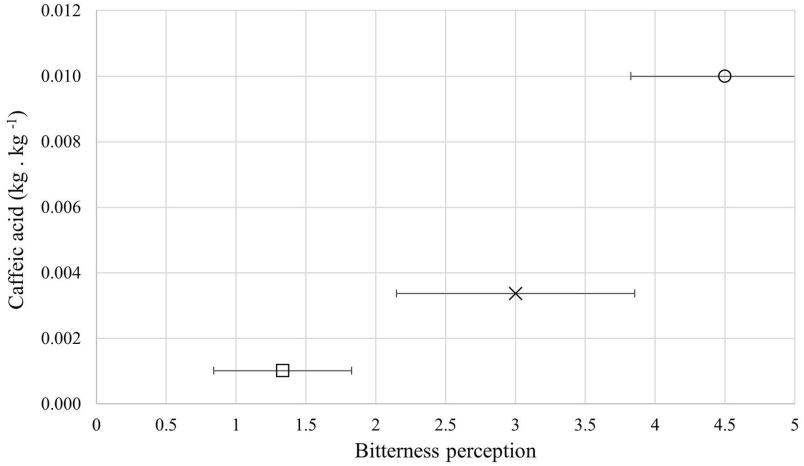

Figure 4: Bitterness perception for black coffee (□), young unfermented coffee leaves (○), and young coffee leave after 160 hours of fermentation with *Aspergillus oryzae* at 30 ºC (x). Values ± standard deviation.

4 DISCUSSION

The enhancement of phenolic profiles on agricultural biomass using *Aspergillus* fermentation have been published with promising results. Most recently, the fermentation of apple waste (with mineral supplementation) was evaluated and showed significant effects on the increase of phenolic contents and antioxidant activities (Gulsunoglu et al., 2020). Other works have used a diversity of biomasses such as mango seeds and black rice to consistently confirm the efficient release of bound phenolics with the consequent enhancement of the antioxidant activity (Shin et al., 2019; Torres-León et al., 2019).

A few publications focused on the biotransformation of coffee biomass with emphasis given only to green coffee beans (Siqueira Palmieri et al., 2018; Soares Bertges et al., 2020). However, our work studied the young leaves since they are considered more abundantly available throughout the year.

In the studies on *Aspergillus oryzae* fermentation of green coffee beans, it was demonstrated an increment of caffeic acid at 24 and 48 h, and correspondently increasing antioxidant activities of the extract. That work obtained a relative 2.9-fold change increase of caffeic acid. However, under those conditions, it was found a total loss of caffeic acid after 72 h. Additionally, the concentration of chlorogenic acid was found to also increase up to 24 h and decrease thereafter.

In agreement with our work, the release of hydroxycinnamic phenolics was attributed to the enzymatic release of bound phenolics, and as the degradation continued glycosides were also hydrolyzed to produce free forms, such as caffeic acid. Nevertheless, our work did not detect increments on chlorogenic acid nor losses of caffeic acid during the period of studies. Similarly, another study on *Aspergillus niger* fermentation of mango seeds observed a 3.3-fold change increase for the total phenolic acid fraction at 20 h and decreasing concentrations afterwards. And for the fermentation of black rice, an increment of free phenolics was found to continue for 3 days up to a 4-5-fold change.

Our study detected a 3.5-fold change increase of caffeic acid at 87 h, which continued rising to 3.7-fold change at 160 h, this was inversely dependent on changes for chlorogenic acid. Thus, the solid-state fermentation of young coffee leaves yielded a final concentration of 0.01403 ± 0.00106 $kg.kg^{-1}$ of caffeic acid as well as 0.01229± 0.00079 $kg.kg^{-1}$ of chlorogenic acid. This biotransformation converted fermented coffee leaves in an enriched source of caffeic acid, with significantly higher concentrations when compared to regularly recognized dietary sources (Meinhart et al., 2019b).

It is very important to consider that our work used "young" *Coffea arabica* leaves, namely the newest 4 leaves, which as determined previously have higher chlorogenic acid concentrations than mature ones (Campa et al., 2017; Chen; Ma; Kitts, et al., 2018; Monteiro et al., 2020). It is generally recognized that a variable range of concentrations are found in different genotypes as well as the maturity stage can highly affect these values (Monteiro et al., 2020). Moreover, agroecological conditions (shade, temperature, altitude) can further determine chlorogenic acid content in coffee (Cheng et al., 2016), as well as different processing techniques can induce compositional changes in coffee leaf tea (Chen; Ma; Kitts et al., 2018).

Chlorogenic and caffeic acid have been well studied as contributing factors of flavor attributes, pointed out as major causes of bitterness and astringency in coffee, beer, or wine. Particularly, it has been found that chlorogenic acid has a bitterness intensity of 2.5 associated with a coffee like bitter taste, while caffeic acid presented an intensity of 5 being associated with harsh bitter taste like strongly roasted coffee (Frank et al., 2007). Our results, in agreement with such studies, found an increased bitterness and astringency for a corresponding increasing caffeic acid concentration. This finding, although not linked to a relative dislike or unpleasant flavor, clearly determined a differentiated quality of fermented samples, which represents a factor to be considered in the development of commercial products.

Other flavor descriptors differentiated fermented and unfermented samples. Unfermented samples were described as having herbal, medicinal taste notes while fermented leaf tea presented honey, and "fermented" like tastes. These qualities clearly distinguished the samples and facilitated the recognition of both during taste analysis.

Although this study did not evaluate the metabolic addition of taste components, it is well recognized that taste is determined by the microbial activity in fermented foods through changes in the composition of amino acids, fatty acids, and organic acids. Certain amino acids have been found to elicit umami (fermented note) as well as sweet taste (He et al., 2019), which in our case could explain the perception of similar descriptors (honey, "fermented like").

5 CONCLUSIONS

The proposed biotransformation of coffee biomass proved to be a highly efficient technique towards the production of caffeic acid enriched foods and could be implemented with relatively simple technology in-farm production systems. Most importantly, this work promoted the use of young coffee leaves visualizing a continual harvesting system with yearlong availability, resembling tea harvesting.

Aspergillus oryzae, isolated from koji rice, induced the cleavage of chlorogenic acid to yield over 3.4-fold change increase of caffeic acid (at 87 hours). The biotransformation was highly selective and induced a chlorogenic:caffeic acid ratio close to 1:1. The resulting contents of caffeic acid were

higher than other reported food sources, which highlights the potential use of this bioprocess for producing an enriched coffee leaf powder, with possible uses as functional food.

Moreover, sensory evaluation of fermented coffee leaves pointed out the effect of the increasing concentration of caffeic acid on the corresponding rise of bitterness and astringency. The potential development of this functional ingredient, which relies on in-farm resources, could include the use of other available bioproducts such as cascara to enhance and optimize flavor in consideration of market niches.

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